WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 6, 1564-1581. Research Article ISSN 2277– 7105

PROTECTION AGAINST EXPERIMENTAL DENTAL CARIES IN RATS WITH CHICKEN EGG YOLK ANTIBODIES (IgY) GENERATED AGAINST *STREPTOCOCCUS MUTANS*

Gandhimathi. C[1*], Sentila.R[2] and Michael A[3]

[1*]Department of Microbiology, Sri Ramakrishna Dental College, Coimbatore, Tamil Nadu, India.

[2,3]Department of Microbiology, PSG College of Arts & Science, Coimbatore, Tamil Nadu, India.

Article Received on
27 March 2015,

Revised on 20 April 2015,
Accepted on 14 May 2015

***Correspondence for Author**
Dr. Gandhimathi. C
Department of Microbiology, Sri Ramakrishna Dental College, Coimbatore, Tamil Nadu, India.

ABSTRACT

High quality oral health care is a vital component of long term general health which plays a major role in the social, economic and personal development of the individual as well as society. Dental caries is the most common infectious transmissible disease in the world today with very high morbidity potential. The prime etiological agent *Streptococcus mutans* plays a crucial role along with dietary carbohydrates in the development of dental caries. Of all the virulence factors of *Streptococcus mutans*, Cell Associated Glucosyltransferase (CA-GTF) enzyme is the most important one in the formation of virulent plaque resulting in initiation and progression of dental caries. Using rat model, evaluation of chicken egg yolk antibodies generated against whole cell antigen and cell associated glucosyltransferase enzyme, as a passive immunotherapy against experimental dental caries was carried out after determining its potential in neutralizing the virulence properties of *Streptococcus mutans* by *in vitro* assays. Tests to detect the ability of antibodies in inhibiting water insoluble glucan synthesis (WIG), and inhibiting biofilm formation in saliva coated hydroxyapatite blocks revealed higher efficacy of antibodies to CA-GTF in inhibiting WIG synthesis both in solution and in biofilm model as well as reduction in the viable count of *Streptococcus mutans* count compared to whole cell antigen of *Streptococcus mutans*. Similarly in rat model in terms of reduction of total *Streptococcus mutans* count and in reduction of caries in rat

molar teeth, antibodies to CA-GTF had greater potential than antibodies to whole cell antigen of *Streptococcus mutans*.

KEYWORDS: Chicken eggyolk antibodies (IgY), Cell Associated Gllucosyltransferase (CA-GTF), Whole cell antigen (WC), Sprague –Dawley Rats (SD Rats).

INTRODUCTION

Oral health, one of the leading healthy indicators for healthy people is an integral part of overall health and has significant impact on general health, and healthy quality life.[1] Of these, dental caries and periodontal diseases are the two important oral health burdens[2] which have been strongly related to socioeconomic factors with the higher incidence in deprived section of the population.[3, 4] Globally the prevalence of dental caries is 60–90% in children and among adults nearly 100% of the population in the majority of countries.[5] Caries levels for 12 year-olds in developing countries has been increasing constantly and this is particularly a cause of concern owing to the fact that the developing countries represent most of our world.[6] As the prevailing measures such as use of fluoride, antiseptic mouth rinse, pit and fissure sealants and floss have not brought down dental caries effectively, the focus is on use of antibodies against cariogenic bacteria as a oral passive immunotherapy against dental caries. Bovine antibodies in milk, immune whey and colostrum against *S.mutans* and *S.sobrinus* significantly inhibited glucan synthesis by *S.mutans.*[7] Mouth rinsing with bovine immune whey by human subjects decreased the relative number of mutans streptococci.[8] Rats provided with the diet containing immune whey had lower plaque scores.[9] Rats fed with Bovine milk containing antibodies against a fusion of the saliva binding alanine rich region of PAc with glucan binding domain of GTF-I showed significant reduction of caries development than controls.[10] Repeated application of monoclonal antibodies against Ag1/11 of *S.mutans* to deciduous teeth of Rhesus monkeys resulted in significant reduction in colonization of *S.mutans* on smooth surfaces and fissures as well as reduction in the subsequent development of caries for a year.[11] Human volunteers who received topical application of monoclonal antibodies had less colonization with *S.mutans.*[12] Recombinant plant monoclonal secretory antibody remained in the oral cavity for 3 days enabling protection for 4 months against oral colonization by *S.mutans*..[13]

Chicken egg yolk antibodies (IgY) does not fix complement and does not bind with Fc receptors of mammalian IgG and hence does not initiate any inflammatory reaction. 25 to 40 gms of IgY can be obtained from single white leg horn hen per year. It does not require any

invasive procedure and involves only simple collection of eggs unlike mammalian source in which bleeding of animals is done. IgY is an alternative promising one among other antibodies. In spite of hyper immune eggs being an excellent, convenient and inexpensive source of IgY, it is still a rarely used resource in medicine. An effective local protection against plaque formation related to dental caries was achieved with anti-*S.mutans* IgY.[14] Administration of IgY against *S.mutans* GBP-B in the form of diet and drinking water in experimentally infected rats caused a significant decrease in *S. mutans* aggregation on dental biofilms and significant reduction in the incidence and severity of dental caries. Even without continuous IgY administration a decrease in *Streptococcus mutans* infection rate was achieved.[15] *S.mutans* is the most cariogenic one with high prevalence in dental carious lesions. *S.sobrinus* has been reported to be prevalent in very low frequency and varies from 0 to 30% in different populations.[16] Only few studies have reported *S.sobrinus* as the sole mutans species, and subjects who have both *S. mutans* and *S. sobrinus* tend to have higher salivary mutans streptococcal counts and severe caries than subjects harbouring *S.mutans* alone.[17]

Adhesins, glucosyltransferases, glucan binding proteins and mutacin are the virulence factors of *S.mutans* of which cell associated glucosyltransferase is the chief virulence factor contributing to the formation of of cariogenic biofilm with heavy aggregation and accumulation of *S.mutans* within a multispecies system.[18] Also in gnotobiotic and specific germ-free rodent models, they have the greatest influence in generating caries.[19] *S.mutans* and *S.sobrinus* have very similar sequences in the functional domains in the catalytic region (CAT) of cell associated glucosyltrnsferae so that antibody against CA-GTF of *S.mutans* can inhibit the activity of GTF of *S.sobrinus*.[20]

In this study chicken egg yolk antibodies generated against whole cell antigen and cell associated glucosyltransferase enzyme were evaluated for their ability in neutralizing virulence attributes of *S.mutans* and *in vivo* efficacy against experimental dental caries in Sprague Dawley rats.

MATERIALS AND METHODS

Chickens: White leghorn chickens (21 weeks old) with known history of immunization and good health conditions purchased from LK poultry farm, Iyyampalayam, (Near Palladam), Coimbatore were maintained in the college layer shed under standard conditions with normal

feeding for the generation of egg yolk antibodies (IgY) against *Streptococcus mutans* whole cell antigen and cell associated glucosyltransferase antigen.

Preparation of Antigens: Standard strain *Streptococcus mutans* serotype *c* 497 obtained from Microbial Type culture collection and gene bank, Chandigarh, India, was used for the preparation of whole cell and cell associated glucosyltransferase enzyme antigens.

Preparation of *Streptococcus mutans* whole cell antigen: *S. mutans c* strain used as antigen was cultured for 24 hours in BHI broth containing 5% sucrose at 37°C under aerobic condition. The culture was treated with 0.5% formalin for 24 hours and then bacterial cells were collected by centrifugation (10000 g for 15 min). The pellets were washed thrice with sterile saline containing 0.5% formalin and resuspended in sterile saline using a Vortex mixer. This formalin killed whole cell antigen samples were kept frozen at -20°C in aliquots until required after the concentration adjustment to 9 $x10^8$ CFU/ml. The antigen was checked for its purity and sterility. Antigen prepared was diluted using sterile saline to a cell concentration of 3 x 10^8 cells.

Immunization with whole cell Antigen of *S.mutans*: 0.5 ml of whole cell antigen was injected intramuscularly at four different sites of breast muscles of chickens. At intervals of 2 weeks 3 more injections of the same dose were given in the same route. Booster doses were given when the antibody titer decreased.

Preparation of cell-associated glucosyltransferase (CA-GTF) antigen: CA-GTF antigen was prepared.[21] and its protein content was determined[22] by using bovine serum albumin (BSA) as the standard. Molecular weight of CA-GTF antigen was determined by SDS-PAGE.[23] and assay of CA-GTF activity was carried out.[24]

Immunization of Hens with CA-GTF antigen: CA-GTF (0.5 ml antigen containing 1mg of protein) antigen suspension was mixed homogeneously with an equal volume of Freund's complete adjuvant for primary immunization and Freund's incomplete adjuvant for booster immunizations. The first immunization was made by injection of 0.5 ml each of the emulsion to the right and left sides of the breast muscle of hens. Booster injections were given with the same route and volume as the first immunization but the FCA was replaced by Freund's incomplete adjuvant. First booster was given on 3rd week, second booster on 7th week and third booster on 11th week.

Serum was collected from chickens before immunization and after immunization at regular specific intervals for detecting presence of specific antibodies and its titer by slide agglutination and micro agglutination test. Eggs were collected before immunization and over a 22 week period after initial immunization and stored at 4°C and processed for isolation of immunoglobulin.

Purification of egg yolk antibodies: The egg yolk antibodies were purified using Pollyethylene glycol (PEG) of different concentrations.[25] IgY extract obtained by the above method was subjected to dialysis.[26] Then it was further purified using DEAE cellulose Ion Exchange Column Chromatography using 25mM phosphate buffer pH 8.0 for binding and 250mM phosphate buffer for eluting IgY. It was then lyophilized and stored at 4˚C for further *in vitro* investigations.

***In vitro* assays:** Molecular weight, protein content, total IgY content, specificity and titer of purified chicken egg yolk antibodies were determined. *In vitro* neutralizing ability was determined by the ability of the antibodies to inhibit water insoluble glucan synthesis in solution and inhibition of biofilm formation on saliva coated Hydroxyapatite blocks in terms of reduction in viable *S.mutans* count and water insoluble glucan synthesis.[31]

Extraction of IgY by water dilution method: The water-soluble fraction (WSF) was isolated from egg yolk. The WSF was prepared using the water dilution method. After separating the yolks from egg white and puncturing the yolk sac with a needle, the contents were allowed to drip through a nylon mesh into a measuring cylinder, and then diluted with 10-fold distilled water. The diluted yolk was adjusted to pH 5.0 with 0.1 N HCl, and stood at 4°C for 24 hours. The solution was centrifuged at 5,500 X g at 4°C for one hour. Disodium hydrogen phosphate (10 mmol/L) was added to the filtrate (water-soluble protein fraction; WSF) and the WSF was adjusted to pH 8.0 with 3 mol/L NaOH. The supernatant was filtered through Whatman No. 52 at 4°, lyophilized and stored at 4°C and was used for animal experiment. Protein content[22] and antibody titer of the IgY extract by ELISA were determined. The purity check of IgY by SDS-PAGE[23] and the estimation of IgY content were carried out.[26]

Selection of Streptomycin resistant strain of *Streptococcus mutans*: Streptomycin resistant strain of *S. mutans* used in this experiment was obtained by one-step selection. This strain was obtained by plating approximately 10^9 cells of a 10 fold concentrated (BHI broth with

0.3% glucose) broth culture of the standard strain on to BHI agar containing 0.2 mg of streptomycin sulfate per ml and incubated aerobically for 48 hours at 37°C. Streptomycin resistant colonies were subcultured in BHI broth and incubated for 2 days. Single colonies were recultured in Brain Heart Infusion Broth and stored in 50% glycerol at -20°C. Immediately prior to the experiment, broth cultures of streptomycin resistant *S. mutans* were streaked onto Mitis salivarius agar plates with or without streptomycin and cultured aerobically for 2 days at 37°C to confirm streptomycin resistance.[15]

Experimental Animals: Animal study was approved by animal ethical committee of PSGIMS Coimbatore (158/99/CPCSEA/182).

21 numbers of Sprague Dawley rats of 19 days old weighing 30 to 35 gm were used for the experiment and were maintained under standard laboratory conditions**.**

Caries induction protocol: Rat chow was pulverized and mixed with 1gram of Ampicillin, 1gram of Carbenicillin, and 1 gram of Chloramphenicol per 1 kg of diet.[27] Rats were fed with the antibiotic diet on days 0, 1 and 2 of experiment to eliminate the normal oral microbial flora of the rats enabling easy establishment of inoculated bacteria in the oral cavity. Oral swabs were obtained from individual rats and cultured on BHI Agar to ensure elimination of microbial flora. The rats at 22 days of age were then infected with streptomycin-resistant (1 mg/ml) strain of *S. mutans* serotype c (MTCC No: 497) by pipette (500 µl of 5 x 10^6 CFU/ml) for 3 days. All the rats were fed throughout the experimental period with modified cariogenic diet (2000).[28] Modified diet 2000 consisted of 56% Sucrose, Skimmed milk powder 28%, Wheat flour 8%, Yeast 5%, Protein powder 2% and Sodium chloride 1%. On 7^{th} day of experiment, oral swabs were taken from all the animals for bacteriological surveys by using a cotton applicator. Samples were vortex mixed and plating was done with appropriate dilutions on agar with 0.2 mg/ml of streptomycin sulfate, and were incubated for 48 hours at 37°C in 10% CO_2 atmosphere for confirmation of the establishment of streptomycin resistant *S. mutans* in the oral cavity and the number of colonies recovered was determined. Bacterial counts were converted to log 10 values.[29]

Experimental group: 19 day old Sprague Dawley rats were divided into 3 groups and put in separate cages comprising of 7 rats at random.

Group A: Control animals were fed on cariogenic diet with non immune IgY 2% and drinking water with 1% non immune IgY from 4th day to 35th day of experiment after which they were fed on cariogenic diet alone.

Group B: Test animals were fed on cariogenic diet with 2% IgY and drinking water with 1% IgY generated against whole cell *Streptococcus mutans* from 4th day to 35th day of experiment after which they were fed with cariogenic diet alone.

Group C: Test animals received cariogenic diet with 2% IgY and drinking water with 1% IgY generated against CA-GTF from 4th day to 35th day of experiment after which they were fed with cariogenic diet alone.

Diet and drinking water were available ad libitum for all the animals.

Recovery of Streptomycin resistant *Streptococcus mutans:* Swabs from all the teeth were taken on 4th, 7th, 15th, 25th, 35th, 45th and 61st day of the experimental period from all animals for enumeration of viable *S.mutans* by using a cotton applicator which was then shaken in a test tube containing 1 ml of sterile phosphate buffered saline, vortex mixed, then diluted in sterile saline (serial 10-fold dilution). Samples of 0.1 ml of appropriate dilutions were streaked evenly with a sterile spreader on the surface of Tryptone soya yeast extract agar incorporated with 20% sucrose, bacitracin and 0.2 mg of streptomycin sulfate /ml and incubated for 48 hours in 5% CO_2 to determine bacterial cell counts. The number of colonies on plates between 50 and 300 colonies was counted. The average counts (three plates per dilution) were used to calculate the total number of CFUs present on the molar teeth of each rat.

Caries assessment: All animals were sacrificed on 61st day of experimental period by ether inhalation. Swabs were taken from all the teeth for bacterial recovery and enumeration. The soft part of the maxilla and mandibles was removed by treating at 125°C for 2 minutes in an autoclave. Mandible and maxilla were removed and stored in plastic containers containing 10% formaldehyde solution. All the molar teeth were examined visually using magnifying lens for any visible lesions. All the teeth were photographed. The molars from each mandible and jaw were embedded in paraffin blocks and sectioned along the sagittal medial-distal plane using hard tissue microtome to the thickness of 100 microns and observed under light microscope to detect the extent and depth of buccal, sulcal and proximal lesions. The caries

induced in all the 12 molar teeth on both maxilla and mandible were scored.[30] Carious lesions were scored based on the depth and breadth of the lesion. Each molar surface is broken down into units corresponding to the cusps of the tooth (4 or 6 units). In each section a lesion or lesions are evaluated and graded based on enamel involvement only (E), Slight dentinal involvement (Ds: up to 25% of dentin involved), moderate dentinal involvement (Dm: 26 to 75% of dentin involved) or excessive dentinal involvement (Dx: over 75% of dentin involved). Scores are recorded for the buccal, sulcal and proximal surfaces individually so that differences among the surfaces can be distinguished. Combined caries scores were determined separately on buccal, lingual, and sulcal dental surfaces. Buccal, lingual, sulcal and proximal surfaces are examined for lesions in enamel, slight dentinal, moderate dentinal and extensive dentinal lesions. When the dentin is extensively involved (Dx) units of penetration are also recorded in the classification of slight (Ds) and moderate (Dm) since units involved in Dx and Dm have necessarily gone through the stages of Ds. Units of involvement diagnosed as Dm are always included under Ds.

Statistical analysis: Differences among means of caries scores obtained from each group of rats were evaluated by an analysis of variance and by multiple mean comparisons using the Duncan test.

RESULTS

***In vitro* assays:** Molecular weight of the GTF enzyme was 160 kDa. Protein concentration was 1.69 mg/ml. Activity of the crude enzyme preparation was 2.4 U/mg of protein. The molecular weight of IgY was 180 kDa. Microagglutination test showed maximum titer of 1:2560 at 49th day. The maximum titer of antibody obtained was 50,000 for both the antigens and the titer was maintained with booster doses for the antibody obtained by both water dilution and polyethylene glycol method**.** Antibody in serum appeared at 4th day and reached its maximum on 21st day and antibody in egg yolk appeared after 1 week and reached maximum on 49th day post-immunization.The protein concentration of anti-WC and anti-CA-GTF IgY generated in egg yolk increased during the immunization period and reached the maximum of 40.61 mg/ml and 40.84 mg/ml respectively. Similarly total IgY concentration reached the maximum of 20.74 mg/ml and 18.72 mg/ml for anti-whole cell IgY and anti-CA-GTF IgY respectively. The maximum protein concentration and total IgY in the fraction obtained by water dilution method were 32.4I mg/ml and 14.52 mg/ml for whole cell antigen and 30.43 mg/ml and 13.21 mg/ml for CA-GTF antigen respectively. Nonimmune IgY

showed 0 reading for specific IgY and with immune IgY there was an increase in the titer of specific IgY by ELISA. Assay of inhibition of water insoluble glucan synthesis by IgY against whole cell and CA-GTF antigens carried out in solution showed inhibition in a dose dependent manner and the percentage inhibition was 64 and 100 respectively at 1 mg concentration. Percentage inhibition of water insoluble glucan synthesis by anti-whole cell IgY and anti CA-GTF IgY in the biofilm model on saliva-coated HA blocks were 58.06 and 93 while inhibition with pre-immune IgY was only 0.4%. [31]

Bacterial recoveries from experimental rats: Colonization with streptomycin resistant *S. mutans* was observed in all animals. In control animals initial colonization was 33 x 10^5 CFU. From 7^{th} day of experiment there was a gradual increase in *S. mutans* count reaching a maximum of $1.27x10^8$. In group B rats fed with immune IgY to whole cell antigen of *S.mutans* initial colonization was $12.6x10^5$ with the final count of $5.2x10^5$ which is significantly less than control group. In group C rats fed with immune IgY to CA-GTF antigen of *S. mutans* initial colonization was $6.2x10^5$ with the final count of 1.6 $x10^5$ which is highly significant than group B. Recolonization of *S.mutans* was less during the treatment period in both the test group.(Table1)

Table1: *Streptococcus mutans* count from dental surfaces of rats during the experimental period and at the end of experiment

Experimental period		7^{th} day	15^{th} day	25^{th} day	35^{th} day	45^{th} day	61^{st} day
Group A(Control)	CFU $x10^5$	33± 1.1	474±11.2	1145±14.5	1208±13.9	1224±16.4	1269±11.1
GroupB (Test)		12.6±0.4	7.6±0.3	7.2± 0.2	6.6 ±0.3	5.8±0.3	5.2±0.2
GroupC (Test)		6.2±0.23	2.4± 0.07	1.31± 0.05	1.52±0.06	1.61±0.05	1.63±0.03

Evaluation and comparison of the effect of IgY to whole cell antigen and CA-GTF antigen on rat caries: Photographed teeth macroscopically showing the presence or absence of caries are presented in the figure1.

Figure 1: Carious lesions in molar teeth of experimental animals

The extent and depth of buccal, sulcal, and proximal lesions demonstrated from light microscopic examination of the sectioned teeth are shown in the figure2. Statistical analysis of caries scores of Enamel, Dentinal slight, Dentinal Moderate, Dentinal severe, smooth surface, sulcal surface, proximal surface and as well as incidence and severity of surface caries, cumulative caries score for individual surfaces and all the surfaces together are given in the tables 2 to 6 and Figures 3 and 4.

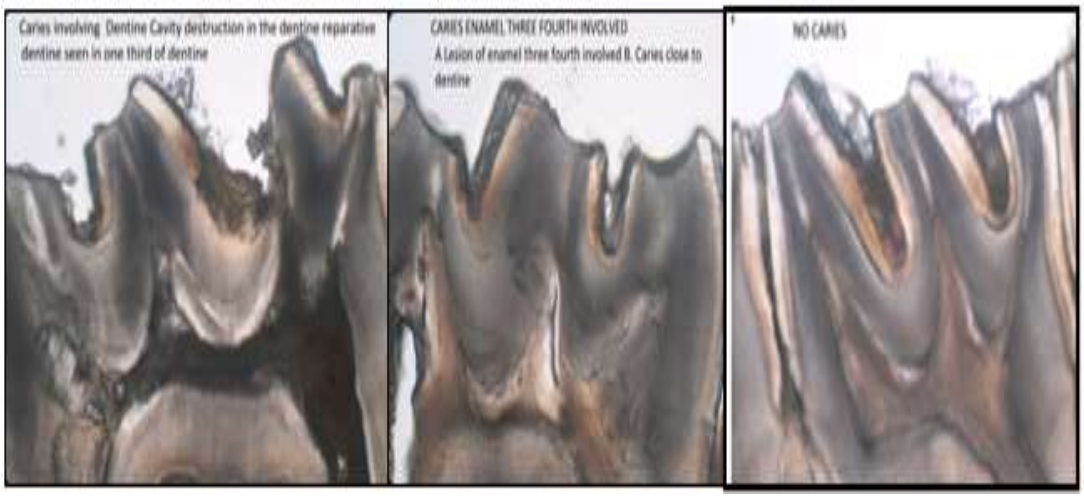


Figure2: Histopathological features of carious lesions

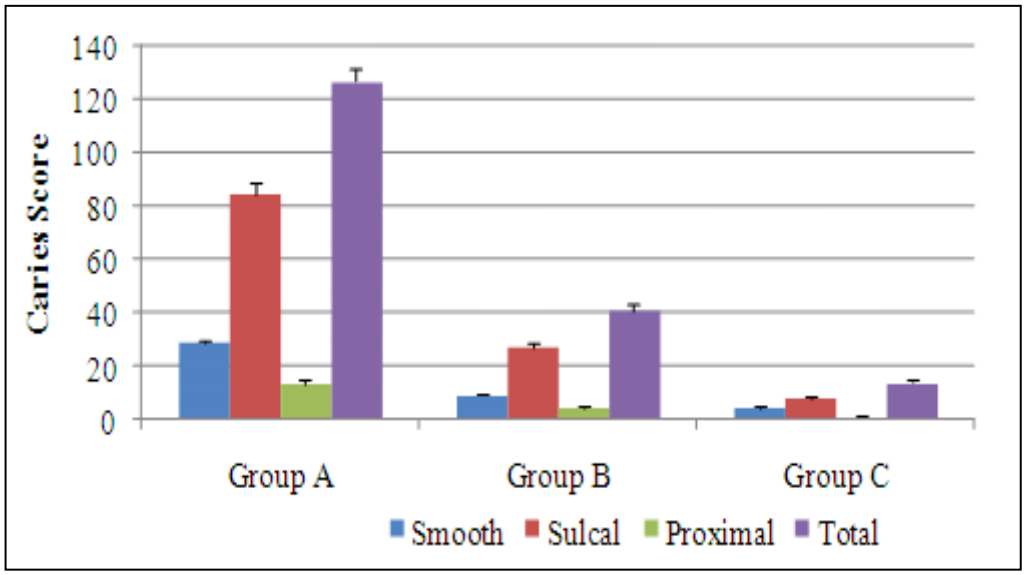
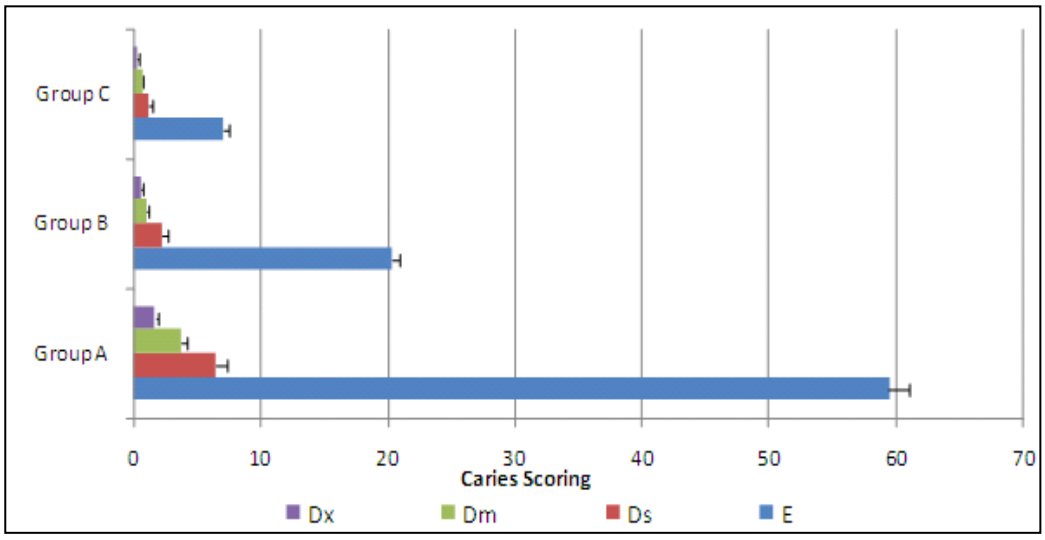


Figure 3: Surface Caries score after treatment between the three study groups



E-Enamel Ds-Dentinal slight Dm-Dentinal moderate Dx Dentinal severe

Figure 4: Severity of the Caries score after treatment between the three study groups

Table 2: Evaluation of Smooth, Sulcal and Proximal surface caries in Rats

		Smooth surface					
Group	**N**	**Mean value**	**Std deviation**	**Mini: mum**	**Maximum**	**F statistics**	**DMRT significance**
Group A	7	28.9	0.89	27	30	2733	c
Group B	7	9.47	0.51	8.9	9.4		b
Group C	7	4.5	0.46		5.2		a
	Sulcal caries						
Group A	7	84.5	4.4	77.5	91.2	1409	c
Group B	7	27.2	1.7	25.4	30		b
Group C	7	8.2	0.86	7.4	9.5		a

	Proximal caries						
Group A	7	13.2	1.6	11.4	15.4	261.5	c
Group B	7	4.1	0.78	3.1	5.3		b
Group C	7	1	0.21	0.1	1.3		a
		Cumulative caries score					
Group A	7	126.8	5.1	117.8	133.4	2228	c
Group B	7	40.8	2.3	37.6	45.3		b
Group C	7	13.7	1.1	12.4	15.3		a

Table 3: Incidence and severity of Enamel and smooth surface caries in Rats

	Smooth surface caries				**Total**
	E	Ds	Dm	Dx	
Group A	16.87 ± 0.44	6.5 ± 0.57	5.82 ± 0.42	1.78± 0.18	28.9±0.89
GroupB	5.54 ± 0.34	2.28± 0.28	1.02 ± 0.21	0.61±0.12	9.47±0.51
GroupC	2.35 ± 0.18	1.21± 0.18	0.68 ± 0.12	0.38±0.12	4.5±0.4

Table 4: Incidence and severity of sulcal surface caries in Experimental Rats

Sulcal caries					**Total**
	E	Ds	Dm	Dx	
Group A	35.65±1.56	24.78±1.39	18.72±1.03	5.4±0.42	84.5±4.4
GroupB	12.05±0.84	7.64±0.41	5.68±0.59	1.85±0.26	27.2±1.7
GroupC	3.94±0.82	2.49±0.32	1.8±0.26	0	8.2±0.8

Table 5: Incidence and severity of proximal caries in Experimental Rats

Proximal caries					**Total**
	E	Ds	Dm	Dx	
Group A	6.91±0.54	3.6±0.53	2.4±0.3	0.81±0.33	13.2±1.6
GroupB	2.75±0.36	1.34±0.38	0	0	4.1±0.78
GroupC	1±0.2	0	0	0	1±0.21

Table6: Evaluation of Dentinal caries severity in Experimental Rats

		Dentinal caries slight					
Group	N	Mean value	Std deviation	Minimum	Maximum	F statistics	DMRT significance
Group A	7	6.50	0.61	5.6	7.3	313.59	c
Group B	7	2.28	0.31	1.9	2.7		b
Group C	7	1.21	0.20	0.9	1.5		a
		Dentinal caries moderate					
Group A	7	3.8	0.46	3.1	4.4	220.68	c
Group B	7	1.02	0.22	0.7	1.3		b
Group C	7	0.68	0.13	0.5	0.9		a

		Dentinal caries severe					
Group A	7	1.786	0.20	1.5	2.1	152.613	c
Group B	7	0.614	0.13	0.4	0.8		b
Group C	7	0.386	0.13	0.2	0.6		a
		Enamel caries					
Group A	7	59.4	1.6	57.2	61.40	F statistics 4047	c
Group B	7	20.3	0.93	19.4	22.1		b
Group C	7	7.1	0.56	6.6	8.1		a

DISCUSSION

Dental caries continues to be highly prevalent in man despite the advances in prophylactic measures and several countries are facing rise of dental caries as a reality which is a cause of serious concern. There is a dire need for the development and broadening of anti-caries measures and the need is a worldwide one. Immunotherapy is one of the appropriate approaches to combat *Streptococcus mutans*, the prime etiological agent of dental caries. Passive immunotherapy against dental caries with chicken egg yolk antibodies is an attractive and effective alternate approach.The present study was conducted to generate Chicken egg yolk antibodies (IgY) by immunizing chickens with whole cell (WC) and Cell associated GTF (CA-GTF) antigens of *Streptococcus mutans* and to evaluate and compare the efficacy of anti-WC and anti-CA-GTF IgY in reduction of both *Streptococcus mutans* as well as dental caries in rat models. *Streptococcus mutans* serotype *c* was used in the present experiment as they are implicated as the most important causative agent in the development of human dental caries. High titer of antigen specific antibody in the egg yolk was observed 7 weeks after immunization in spite of high titer noted in serum of chickens at third week after immunization. *Streptococcus mutans* count in rat molars observed at the end of the experimental period showed a significant difference between the control and test groups which revealed that anti-CA-GTF IgY is relatively more efficient in preventing the accumulation of *S.mutans* compared to anti-whole cell IgY. In the control animals more carious lesions were observed in mandibular molars than maxillary molars similar to the observation of Sognnaes. R.F. (1949)[32] and second molar tooth was seen to be completely destroyed in few control rats. Carious lesions were more in sulcal surface than smooth surface of the molars and very few lesions were observed in proximal surface of control rats. Statistical analysis showed significantly less enamel carious lesions in both group of rats (group B) fed with anti-whole cell IgY (mean value 20.3±0.93) and (group C) anti-CA-GTF IgY (mean value 7.1±0.56) when compared.with the control group (mean value 59.4±1.6). But the results of the group fed with anti-CA-GTF IgY were highly significant. Similarly in

dentinal caries of slight, moderate, severe as well as smooth, sulcal, proximal and total, the efficacy of anti whole cell IgY and anti CA-GTF IgY in inhibiting the development of carious lesions were found to be significant. Among the test groups, mean caries score in rats on IgY to CA-GTF diet was significantly less than group B. The percentage inhibition of enamel, dentinal slight, dentinal moderate, dentinal severe, smooth, sulcal, proximal surface and total all surfaces caries in group fed with anti-whole cell (groupB) and anti-CA-GTF IgY (group C) was calculated with mean caries of control (group A) fed on pre-immune diet. It is evident from the inhibition percentage of enamel caries (score of 65.95% in group B and 88.1% in group C), that despite the efficacy shown by both the immune IgY in preventing the development of enamel carious lesions, immune IgY to CA-GTF is obviously more effective than immune IgY to whole cell antigen of *Streptococcus mutans.* Dentinal slight (Ds), dentinal moderate (Dm), and dentinal severe (Dx) carious lesions were less in both the groups with a marked inhibition in caries development in group C. The extent of inhibition in % were 65 (Ds), 73.2 (Dm) and 65.7 (Dx) in group B and 81.4 (Ds), 82.1 (Dm) and 78.4 (Dx) in group C which again shows higher efficacy of anti CA-GTF IgY in protection against carious lesions. Similarly with regard to smooth surface, sulcal surface, and proximal surface carious lesions, the percentage inhibition in group B was 67.5, 68 and 69 and in group C was 84.5, 91.3, and 92.5 respectively. Enamel and severity of lesions of smooth surface, sulcal surface and proximal surface were significantly less from which it is very clear that IgY to CA-GTF plays a pivotal role in inhibiting the development of dental surfaces carious lesions. When all the surface lesions together were taken into account the inhibition observed in rats treated with immune IgY to whole cell and CA-GTF was 67.9% and 89.2% respectively. Rats fed on cariogenic diet supplemented with immune IgY to CA-GTF of *Streptococcus mutans* showed significantly very low *Streptococcus mutans* count and few carious lesions compared to the rats fed with cariogenic diet incorporated with immune IgY to whole cell *Streptococcus mutans.* In a study by Hamada *et al*., in 1991[33] with the use of 1% immune IgY to CA-GTF, there was 46% inhibition in total caries score but the present study shows inhibition of 82.6% with 2% immune IgY which is highly significant. The study of Hamada *et al.,* (1991) have not shown significant inhibition in caries development in rats fed with immune IgY to whole cell antigen which is contradictory to the result obtained in this study with anti-WC IgY. Otake *et al*., (1991)[14] have shown inhibition of 60% in total caries development by incorporating water soluble fraction of immune IgY to whole cell *Streptococcus mutans* for 56 days which correlates with the present study result. Kruger *et al*., (2004)[34] infected rats with *Streptococcus mutans* MT8148R and then treated the rats with chicken anti-CA-GTF

egg yolk antibodies (IgY) and found significant inhibition in the development of smooth surface lesions in the anti-CA-GTF-treated group in comparison to the control groups. Sulcal surface caries was also less and was of less severity. These results parallel with the findings of the present study which showed significant inhibition of smooth as well as sulcal lesions occurrence in rats. In the studies by Ma and co-workers in1990[35] and 1998[13], rats exposed to monoclonal antibody were protected for long duration from developing caries even after the supplemented antibody was stopped. In those studies, rats were treated with topical antibacterial mouthwash after which they were exposed to mouse or plant antibodies to *S.mutans* antigen 1/II adhesion during *Streptococcus mutans* recolonization. In the study conducted by Daniel J. Smith *et al*., (2001)[15] immune IgY to Glucan binding protein-B was given to rats for 9 days and there was not much statistical significance in the accumulation of *S. mutans* even though accumulation was at lower level compared to control animals. In the present experiment immune IgY was given for 33 days which includes the period of the eruption of third molars. The third molars of the rats were not colonized with *Streptococcus mutans* compared to other two molars in whom implantation of *Streptococcus mutans* was done in the presence of antibodies in case of test animals and without antibody in control animals and third molars also had the benefit of inhibitory dietary IgY antibody. Third molars were more readily protected than other two molar surfaces. These studies support the use of oral passive immunization during the window period of infectivity. The results of the present rat model study clearly supports passive immunization with immune IgY to CA-GTF as most efficient in inhibiting the accumulation of *Streptococcus mutans* on teeth surfaces and thereby preventing the development of dental caries effectively. As obtaining chicken egg yolk antibodies is noninvasive and there is no dearth of its sustainable availability along with the large yield of 25 to 40gms/hen/year and its effectiveness against concerned bacteria immuotherapy with specific IgY is a desirable option for elimination of dental caries.

CONCLUSION

In vitro studies and rat model have revealed that Chicken egg yolk antibodies against CA-GTF can be utilized for effective prevention of dental caries and clinical trials are needed for successful application of egg derived antibodies.

CONFLIT OF INTEREST: The authors declare that they have no conflict of interests.

REFERENCES

1. Petersen, P.E,.The World Oral Health Report; continuous improvement of oral health in the 21st century - the approach of the WHO Global Oral Health Programme. Community Dent. Oral Epidemio., 2003; 31(Suppl 1): 3-24.
2. Petersen, P.E. and H.Ogawa, 2005. Strengthening the prevention of periodontal disease: the WHO approach . Journal of Periodontal Research, 2005; 76(12): 2187-2193.
3. Beaglehole R., H. Benzian, J.Crail and J.Mackay, 2009. The oral health atlas: mapping a neglected global health issue. Geneva and Brighton: FDI World Dental Education Ltd and Myriad Edition.
4. Sweeney P.C., Z. Nugent and N.B., 1999. Deprivation and dental caries status of 5 year old children in Scotland. Community Dentistry and Oral Epidemiology, 1990 ; 27: 152-159.
5. Marcenes W., N.J. Kassebaum, E. Bernabé, A. Flaxman, M. Naghavi, A.Lopez and C.J. Murray. Global burden of oral conditions in 1990–2010: a systematic analysis. J. Dent. Res., 2013; 92(7): 592–7.
6. World Health Organization Oral health Fact sheet N°318 April 2012.
7. Loimaranta, V., J. Tenovuo, S. Virtanen, P. Marnila, E. L. Syväoja, T.Tupasela, and H. Korhonen. 1997. Generation of bovine immune colostrums against *Streptococcus mutans* and *Streptococcus sobrinus* and its effect on glucose uptake and extracellular polysaccharide formation by mutans streptococci. Vaccine, 1997; 15: 1261–1268.
8. Loimaranta V., M. Laine, E. Soderling, E. Vasara, S. Rokka and P. Marnila, H.Korhonen O. Tossavainen and J. Tenovuo. Effects of bovine immune and non-immune whey preparations on the composition and pH response of human dental plaque. Eur. J. Oral. Sci., 1999; 107(4): 244-250.
9. Michalek, S. M., R. L. Gregory, C. C. Harmon, J. Katz, G. J. Richardson, T.Hilton, S. J. Filler, and J. R. McGhee. 1987. Protection of gnotobiotic rats against dental caries by passive immunization with bovine milk antibodies to *Streptococcus mutans*. Infect. Immun., 1987; 55: 2341–2347.
10. Mitoma M., T. Oho, N. Michibata, K. Okano, Y. Nakano, M. Fukuyama and T.Koga, 2002. Passive immunization with bovine milk containing antibodies to a cell surface protein antigen - Glucosyltransferase fusion protein protects rats against dental caries. Infect. Immu., 2002; 70: 2721–2724.

11. Lehner T., J. Caldwell and R. Smith, 1985. Local Passive Immunization by Monoclonal Antibodies against Streptococcal Antigen I/II in the prevention of dental Caries. Infection and Immunity, 1985; 50(3): 796–799.
12. Ma, J.K.C., R. Smith, and T. Lehner. Use of Monoclonal Antibodies in Local Passive Immunization to Prevent Colonization of Human Teeth by *Streptococcus mutans*. Infection and Immunity, 1987; 55: 1274-1278.
13. Ma, J. K., B. Y. Hikmat, K. Wycoff, N. D. Vine, D. Chargelegue, L. Yu, M. B. Hein, and T. Lehner. Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. Nat. Med., 1998; 4: 601–606.
14. Otake, S., Y. Nishahara, M. Makinura, H. Hatta, M. Kim, T. Yamamoto, and T. Hirasawa. Protection of rats against dental caries by passive immunization with hen-egg yolk antibody (IgY). J. Dent. Res.. 1997; 70: 162–166.
15. Smith D.J., W.F. King, and R.Godiska. Passive transfer of immunoglobulin Y antibody to *Streptococcus mutans* glucan binding protein B can confer protection against experimental dental caries. Infect. Immun., 2001; 69(5): 3135-3142.
16. Lindquist, B. and C.G. Emilson. 1991. Dental location of *Streptococcus mutans* and *Streptococcus sobrinus* in humans harboring both species. Caries Res., 1991; 25: 146–152.
17. Kohler B., I.Andreen and B.Jonsson. 1988. The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age. Oral. Microbiol. Immunol., 1988; 3: 14-17.
18. Tamasada M., S. Kawabata, T. Fujiwara and S.Hamada. Synergistic effects of streptococcal glucosyltransferases on adhesive biofilm formation. J. Dent. Res., 2004; 83: 874–879.
19. .Takahashi, N. and B. Nyvad, Caries ecology revisited: microbial dynamics and the caries process. Caries Res., 2008; 42: 409–418.
20. Jespersgaard C., G. Hajishengallis, T. E. Greenway, D. J. Smith, M. W. Russell and S. M. Michalek, Functional and immunogenic characterization of two cloned regions of *Streptococcus mutans* glucosyltransferase. Infect. Immun., 1999; 67: 810-816.
21. Hamada, N. and HK.Kuramitsu,1989. Isolation and characterization of the *Streptococcus mutans* gtfD gene, coding for primer-dependent soluble glucan synthesis. Infect. Immun., 1989; 57: 2079–2085.
22. Lowry O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. Protein measurement with the folin-phenol reagent. J.Biol.Chem., 1951; 193: 265 – 275.

23. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970; 227: 680-685.
24. .Al-Hebshi N.N., Q. Neilsen. and N. Skaug. In vitro effects of crude khat extracts on the growth, colonization, and glucosyltransferases of *Streptococcus mutans.* Acta. Odontologica Scandinavica., 2005; 63(3): 136–142.
25. Polson, A., M. B. von Wechmar, and G. Fazakerley. Antibodies to proteins from yolk of immunized hens. Immunol. Commun., 1980; 9: 495-514.
26. Pauly D., P.A. Chacana, E.G. Calzado, B. Brembs and R. Schade, IgY Technology: Extraction of Chicken Antibodies from Egg Yolk by Polyethylene Glycol (PEG) Precipitation. Journal of Visualized Experiment., 2011; 51(e3084): 1-5.
27. Michalek., S. M, and J. R. McGhee. 1977. Virulence of Streptococcus mutans: an antibiotic- suppressed rat model for studies of pathogenesis. J. Dent. Res., 1977; 56: 205-211.
28. Regolatti, B. and M. uhleman1971. Effect of urease inhibition on calculus formation and caries development in the rat. Helv. Odont. Acta., 1971; 15: 151–154.
29. Keyes, P.H. and R.J. Fitzgerald. Dental caries in the Syrian hamster. IX. Arch Oral Biol., 1962; 7: 267–277.
30. Keyes, P.H. Dental caries in the molar teeth of rats. II. Amethod for diagnosis and scoring several types of lesions simultaneously. J. Dent. Res., 1958; 37: 1088-1099.
31. Gandhimathi, C. and A. Michael, 2014. *In vitro* neutralization of virulence properties of *Streptococcus mutans* using chicken eggyolk antibodies (IgY) Int J Pharm Bio Sci., 2014; 5(2): (B) 498 – 508.
32. Sognnaes, R.F., 1949. Experimental rat caries; location, sequence and extent of carious lesions produced in the Norway rat when raised on a generally adequate, finely powdered, purified ration. *The Journal of nutrition*, 1949; 39(2): 139-146.
33. Hamada S., T. Horikoshi, T. Minami, S. Kawabata, J. Hiraoka, and T. Fujiwara, Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of *Streptococcus mutans*. Infect. Immun., 1991; 59: 4161-4167.
34. Kruger C., SK. Pearson, Y. Kodama, A. Vacca Smith, W.H. Bowen and L Hammarstrom. The effects of egg-derived antibodies to glucosyltransferases on dental caries in rats. Caries Res., 2004; 38(1): 9-14.
35. Ma, J.K. and T. Lehner. Prevention of colonization of *Streptococcus mutans* by topical application of monoclonal antibodies in human subjects. Arch. Oral Biol., 1990; 35: 115-122.